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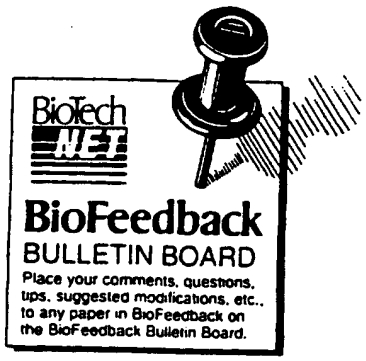

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Dual Asymmetric PCR: One-Step Construction of Synthetic Genes

ABSTRACT

We have developed a one-step process for constructing synthetic genes. Four adjacent oligonucleotides 17–100 bases in length having short overlaps of 15–17 bases are used as primers in a PCR mixture. The quantity of the two internal primers is highly limited, and the resultant reaction causes an asymmetric single-stranded amplification of the two halves of the total sequence due to an excess of the two flanking primers. In subsequent PCR cycles, these dual asymmetrically amplified fragments, which overlap each other, yield a double-stranded, full-length product.

INTRODUCTION

In this paper we describe a novel, but general, process for *in vitro* construction of a double-stranded DNA. Specifically, we used the technique to synthesize 220 base pairs (bp) of DNA encoding the hirudin gene. Hirudin is a 65-aminoacyl protein that binds to thrombin, thereby preventing blood coagulation (4,5). Previously published methods for *in vitro* double-strand DNA synthesis using synthetic oligonucleotides consisted of multiple steps and are more labor-intensive than the method described herein. One method consists of using purified, phosphorylated, overlapping oligonucleotides to "block-assemble" a fragment of about 600 bp (9). The correctly assembled DNA is usually a minor species and must be purified from the undesired species.

With the advent of PCR (8), the labor of *in vitro* gene synthesis has been reduced but published methods still are more tedious than is necessary. For example, PCR was used to amplify the correct block-assembled, double-strand DNA using the terminal oligonucleotides as primers (3). In another approach, extremely long oligonucleotides up to 254 bases were synthesized *in vitro*. Because long oligonucleotides are produced in very low yields, subsequent PCR amplification with tw flanking primers is needed to obtain reasonable yield of the desired duple product (1). The first two methods ar maximally expensive because bot strands are synthesized *in vitro*. Addi tionally the last method is not ver practical because very large oligo nucleotides are difficult to make.

The problem of costs can be re duced by using complementary oligo nucleotides that overlap on their 3 ends in combination with enzymatic fill-in to complete production of the double-stranded DNA. Mullis et al. (6 used this tactic to produce a duplex. This duplex was then annealed with two more oligonucleotides that overlapped its opposite ends to progressively grow a large duplex of 374 bp. The 374-bp duplex was amplified by PCR to realize a usable amount of product. Their procedure is also a multi-step process. In the current paper we describe a procedure that is similar to that of Mullis et al. except that it is accomplished in one step.

MATERIALS AND METHODS

A synthetic gene for hirudin was synthesized using the following protocol. Four oligonucleotides were synthesized based on the known hirudin protein sequence and the codon usage in *E. coli* (Figure 1). Primer H1 (nucleotides 1–86), primer H2 (nucleotides 149–72), primer H3 (nucleotides 138–220) and primer H4 (nucleotides 220–204) were synthesized on Applied Biosystems 394 RNA/DNA synthesizer (Foster City, CA) and gel purified.

H1 and H4 (12.5 pmol), along with 0.125 pmol of H2 and H3, were put in 25 µl of a standard PCR mixture (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each deoxynucleoside triphosphate [dNTP] and 1 unit *Taq* DNA Polymerase, Perkin-Elmer Cetus, Norwalk, CT). Twenty-five to 35 cycles were carried out at 94°C for 1 min, 50°C for 2 min and 72°C for 3 min. Primer H1 has an *NdeI* site at its 5′ end while primer H4 has an *EcoRI* site at its 3′ end which enabled the cutting of the fragment with *NdeI*/*EcoRI* and subsequent cloning into the *E. coli* expression vector pET-3b (7).

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RESULTS AND DISCUSSION

We reasoned that if four overlapping oligonucleotides were mixed and annealed in the presence of buffer, nucleotides and *Taq* polymerase, then the reactions shown in Figure 1 would occur under PCR conditions. By using an asymmetric ratio of oligonucleotides in which the two internal oligonucleotides, H2 and H3, are limiting, we bias the system to make double- and single-strand intermediates of the type shown in Figure 1, step b. These intermediates anneal and prime the synthesis of full-length, double-strand molecules (step c) that can then be amplified (step d) because of excess H1 and H4 oligonucleotide primers.

To see if the intermediates predicted in Figure 1 occurred with the kinetics postulated, we set up a series of identical reactions and analyzed them after a total of 5, 10, 15, 20 and 25 cycles of amplification. The results are seen in Figure 2. Four lower molecular weight intermediates appear in lanes 1 to 5, two just above the 72-bp marker and two between the 118- and 194-bp markers. The two smaller bands represent a mixture of the double-strand, 83 bp H4 primed duplex, excess single strands primed by H4 and unincorporated primers H1, H2 and H3. Unfortunately, we cannot identify the exact composition of either band. The important point to note is all species disappear as PCR proceeds beyond 10 cycles, while the 220-bp duplex concomitantly increases after 10 cycles.

The pair of bands between duplex markers 118 and 194 bp must represent the postulated 149-bp duplex and single-strand intermediates amplified from it. They have a quasi-equilibrium concentration between the 5th and 10th cycles and largely disappear by the 15th cycle. Also, by 15 cycles the 220-bp duplex is approaching a maximal amount.

Under our reaction conditions a minor, larger species also appears around 305 bp. We do not know what it is. Possibilities are spurious priming or an electrophoresis artifact. Its existence does not alter the fact that our method works efficiently as verified below.

The reaction run in Figure 2 was repeated and the 220-bp fragment excised and cloned. Two isolates were analyzed by DNA sequencing and found to have the sequence of the component oligonucleotides (data not shown). This observation validates our supposition that the major final product is as predicted in Figure 1. An added proof is the fact that after we transferred the cloned hirudin sequence to an expression vector, it produced a thrombin inhibitor in *E. coli* host cells (B. Owen, personal communication).

Synthetic oligonucleotides 100 bases long can be routinely synthesized in reasonable yields. Using our method with 100-mers having 15-bp overlaps, a 355-bp duplex could be generated. If two 355-mers overlapping by 15 bp were made in separate reactions and then mixed, in principle they could be

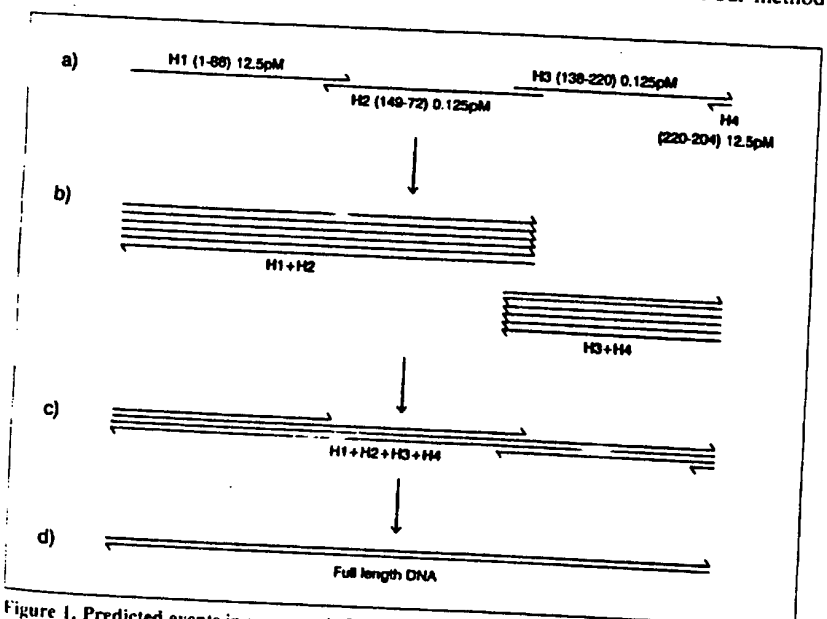

Figure 1. Predicted events in asymmetric PCR amplification. a) Synthetic hirudin oligonucleotides showing their relative positions, lengths, regions of complementarity and concentrations at the beginning of the PCR. b) Limited primers H2 and H3 are rapidly depleted in the initial cycles, following which the 100-fold excess of primers H1 and H4 causes an excess of single strands H1+H2 and H3+H4 to be synthesized. c) These dual asymmetrically produced single strands overlap and are filled across in subsequent cycles resulting in the synthesis of a full-length, double-stranded synthetic gene. Primers H1 and H4, along with the asymmetric single strands, increase the yield by further amplifying this full-length template. d) On completion of reaction, nearly all oligonucleotides get incorporated into full-length, double-stranded DNA (see Figure 2, lane 5).

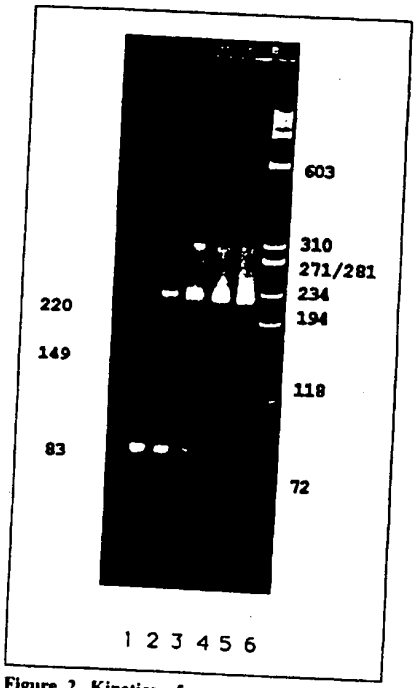

Figure 2. Kinetics of asymmetric amplification of hirudin gene. The four primers in the asymmetric amounts shown in Figure 1 were mixed with buffer, nucleotides and enzyme, subdivided into five separate test tubes and placed in a thermocycler. A tube was pulled after each five cycles of amplification, and the contents analyzed by electrophoresis in a 5% polyacrylamide gel. Thus, material in lane 1 is the product of five cycles of amplification, lane 2, 10 cycles, etc. The markers are an *Hae*III digest of ϕX174 duplex DNA.

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amplified as a continuous 695-mer using the appropriate rightmost and leftmost priming sequences (2). Thus, one could grow a synthetic gene of quite large proportions ultimately being limited by the effective distance *Taq* polymerase can amplify under PCR conditions. The overall advantages of our method are a reduction in steps and time needed to make duplex oligonucleotides in the size class of 200 to 350 bp and an approximate 40% savings in oligonucleotide costs compared with the "block" method of assembly. Clearly, it also represents a straightforward way for production of genes with any mutation desired.

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Amplification of Reproducible Allele Markers for Amplified Fragment Length Polymorphism Analysis

ABSTRACT

A procedure for amplification by PCR of reproducible allele markers for amplified fragment length polymorphism (Amp-FLP) analysis is presented. We have prepared markers for the allelic products of the VNTR loci D1S80 (MCT118) and D17S30 (YNZ22) and for the hypervariable VNTR locus close to the 3′ end of the apolipoprotein B gene (apoB) by re-amplifying a mixture of PCR products from individuals with known alleles. These allele markers allow precise and discrete determination of the VNTR alleles at these loci using the Amp-FLP technique that should prove suitable in forensic analyses, paternity testing and population studies.

INTRODUCTION

The highly polymorphic variable number of tandem repeat (VNTR) loci (7) in the human genome are useful markers for genetic characterization of individuals in forensic science and paternity testing. The amplified fragment length polymorphism (Amp-FLP) technique is a new convenient method to analyze VNTR loci (3). Using the Amp-FLP technique, relatively small-sized (<2 kb) VNTR loci are amplified by PCR (8), and the amplified DNA fragments are separated by high-resolution polyacrylamide gel electrophoresis (PAGE). Subsequent to electrophoresis the DNA fragments are visualized by silver staining. The Amp-FLP technique can resolve DNA fragments differing by less than 10 bp (1). Therefore, it is possible to separate alleles discretely at a number of VNTR loci. In contrast to classical restriction fragment length polymorphism (RFLP) analysis, more conventional approaches for assessing geno/phenotype distributions and Hardy-Weinberg expectations in a population sample can be applied (3,4). The determination of discrete alleles enables more effective comparisons of samples in forensic casework than can be achieved by RFLP analysis and the present limitations of electrophoresis.

Reliable and easy identification of the Amp-FLP alleles requires a marker comprising a representative number of alleles for each VNTR locus. Such an allele marker could be generated by amplifying individual genomic DNA samples from persons with known alleles, followed by mixing the PCR products to produce a composite marker. Individual genomic DNA samples can also be first mixed and then amplified to produce an allele marker. However, these approaches are expensive, time-consuming, and may eventually deplete the genomic DNA sources. Alternatively, cloning of the ladder fragments would provide an unlimited source of allele markers, but this is laborious and requires facilities for recombinant DNA work.

Multiplex amplification demonstrates that DNA fragments of different sizes can be amplified simultaneously in one reaction (5). Furthermore, according to our previous experience from analysis of alleles at the hypervariable region close to the 3′ end of the apolipoprotein B gene (apoB), the apoB locus can be re-amplified without production of spurious PCR products (10). Therefore, an allele marker consisting of PCR products for a VNTR locus should be amenable to re-amplification by PCR. Re-amplification of an allele ladder is simple, inexpensive and would enable worldwide standardization of the Amp-FLP technique for identity testing. The virtually infinite supply of allele markers allows distribution of the same allele markers to forensic/paternity laboratories, and each laboratory can then generate its own supply by re-amplification of the allele marker previously produced by PCR.

In the present paper we describe the feasibility of re-amplification of allele ladders for Amp-FLP analysis of D1S80 (MCT118) (6) and D17S30 (YNZ22) loci (12) and the hypervariable region close to the 3′ end of the apoB gene (2).

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